BIOCHEMICAL CORRELATES OF DISCONTINUOUS MUSCLE REGENERATION IN THE RAT

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EXTENSIVE studies have been made by many workers, with the aid of conventional histological methods (Field, 1960), autoradiographic procedures (Bintliff and Walker, 1960) and, more recently, by using electron microscopic techniques (Allbrook, 1962 ; Price, Howes and Blumberg, 1964*a*, *b*) of the cells involved in the regeneration of skeletal muscle fibers, after various types of muscle damage.

The comparative biochemistry of developing and mature skeletal muscle (Davidson and Waymouth, 1944 ; Leslie and Davidson, 1951 ; Robinson, 1952*a*, *b* ; Hartshorne and Perry, 1962 ; Perry and Hartshorne, 1963) has so greatly added to our knowledge of pre- and post-natal muscle growth, that further characterization, by biochemical methods, of the changes accompanying the regeneration of injured skeletal muscle fibres, seem desirable. So far, however, biochemical investigations have not been carried out on regenerating skeletal muscle, owing to the limited yield of tissue available for chemical or enzymatic analysis by most methods used to study muscle regeneration, *e.g.* ischaemic, cold, and crush injury of skeletal muscle fibres.

The literature relating to alternative experimental procedures for obtaining extensive regeneration of skeletal muscle, and our own experience (Aloisi, Ferolla and D'Ancona, 1963) with the method developed by Studitskji (1959) from the original techniques of Le Gros Clark (1946), has led us to consider muscle autografts as a suitable system in which to study the biochemical correlates of muscle regeneration. The whole gastrocnemius muscle can be removed from the leg of the rat. Extensive regeneration of muscle fibres is obtained, according to a definite time-course of cellular events, on grafting the mince prepared from the same muscle in its original anatomical area. Furthermore, samples of the regenerating tissue can be disposed of in quantities which are within the reach of available methods of chemical and biochemical analysis (Aloisi, Ferolla and D'Ancona, 1963). In the course of additional histological investigations with regenerating rat gastrocnemius, to be reported in detail elsewhere (Aloisi, unpublished), the observation has also been made that the regeneration of muscle fibres is positively influenced by agents capable of blocking the maturation of fibrous collagen, such as the lathyrogen aminoacetonitrile.

To provide information on the pattern of growth of autologous grafted minces of skeletal muscle, in untreated and aminoacetonitrile (AAN) treated rats, changes in the protein composition, relating to the formation of contractile proteins, and changes in the nucleic acid concentration of the regenerating tissue, are presented here.

To glean some further insight on the degree of development attained by the regenerating skeletal muscle fibres under the above outlined experimental conditions, the activity profile of several enzymes associated with carbohydrate metabolism has also been determined in regenerating muscle, and has been compared with that of developing (neo-natal) and mature skeletal muscle.

MATERIALS AND METHODS

Animals and animal care.—Male Wistar rats of about 150 g. body weight were used. They were fed a stock laboratory diet. Some of the animals were administered daily AAN, dissolved in physiological saline, at the dose level of 50 mg./kg. body weight, by intraperitoneal injection. The treated rats generally exhibited only mild signs of toxicity after receiving AAN for as long as two months. A few animals, however, died in the course of the treatment. Control rats received no treatment.

Surgical intervention.—This was patterned on the experimental procedure of Studitskji (1959). Rats were anaesthetized with avertine (Bayer). The gastrocnemius muscle of one side was dissected with a minimum of trauma from its surrounding connective sheath and from the subjacent soleus muscle and sciatic nerve. After being removed from the animals by trans-secting the Achilles tendon and cutting its proximal attachment as close as possible to the bone, the whole muscle was finely minced with scissors in a sterile Petri-dish. About 2/3 of the tissue mince could be accommodated in the anatomical lodge originally occupied by the intact gastrocnemius. The muscle layer formed by the biceps and then the skin were separately sutured over the graft.

Light microscopy.—Specimens of tissue were fixed in formalin-mercuric chloride fluid. Blocks were embedded in paraffin, cut at 8 μ and stained with haematoxylin-eosin or by the AzanMallory method.

Analytical Procedures

Treatment of tissue.—The entire regenerating muscles from 2–4 rats (300–600 mg.), were combined, finely minced with scissors and weighed. The mince was thoroughly mixed in order to allow sampling of amounts of the same composition. Portions of about 100 mg. and 250 mg. were weighed out and used for nucleic acids and protein analysis, respectively. In some experiments, additional amounts of tissue were used for enzymatic analysis. Homogenates (final concentration 10 per cent) were prepared from the tissue with a Potter-Elvehjem (1936) apparatus in 5 mM Tris (hydroxymethyl) aminomethane (Tris) — 1 mM ethylenediaminotetraacetic acid (EDTA), pH 7·4, or in 20 mM K-phosphate buffer pH 8·0. Enzyme activity determinations were made on the supernatant fraction obtained by centrifuging the tissue homogenates at 10,000 × **g** for 10 min.

Fractionation of proteins.—Weighed samples of minced tissue were extracted with salt solution of the following composition: 1·25 M-KCl, 0·066 M-K_2HPO_4, pH 8·5, I = 1·45. Fibrillar proteins were precipitated from the extract by adding 0·066 M-$NaHPO_4$ and water, and further diluting with 19 per cent ethanol to a final ionic strenth of 0·12, essentially as described by Robinson (1952, *a*). The residue was treated with 0·1 N-NaOH, and the extract was combined with the fibrillar protein fraction. The two combined fractions will be referred to as " contractile proteins ", (" fibrillar proteins fraction " of Robinson). Collagen (insoluble connective tissue) was extracted from the residue after alkaline treatment by autoclaving in the presence of 6 ml. of water (Jackson, 1957). Duplicate aliquots of the gelatin solutions obtained were hydrolysed with concentrated HCl in sealed tubes (Neuman and Logan, 1950). The amount of collagen was estimated from hydroxyproline analysis of the neutralized hydrolisates, by a modification (Martin and Axelrod, 1953) of the method of Neuman and Logan (1950). Hydroxyproline values were converted into mg. of collagen by using a factor of 7·46.

Determination of nucleic acids.—Nucleic acids were isolated by treating aliquots of 10 per cent tissue homogenates in distilled water according to the Schneider procedure (1957), under the conditions described previously (Margreth and Novello, 1964). For measurement of RNA or of total nucleic acid phosphorus, the dry, defatted ribonucleoprotein residue was extracted with 5 per cent trichloroacetic acid, at 90° for 15 min. When DNA was determined, the hot acid extraction was carried out with 1 N perchloric acid, at 70° for 20 min.

The nucleic acid phosphorus was estimated by digesting aliquots of the nucleic acids extracts with 10 N–H_2SO_4, essentially as described by Le Page (1959), and by determining inorganic phosphate by the method of Ernster and Lindberg (1955). RNA was estimated from ribose analysis by the orcinol reaction (Schneider, 1957). DNA was estimated from deoxyribose analysis according to Burton (1956).

Enzymatic assays.—Glycogen phosphorylase activity was measured on Tris-EDTA extracts, further diluted with 20 mM Na F–1 mM EDTA, in the absence and in the presence of 1 mM AMP at pH 6·0, according to the procedure of Cori, Illingworth and Keller (1955). The liberation of inorganic phosphate from glucose-1-phosphate was determined on amounts of the reaction mixture by the method of Ernster and Lindberg (1955).

Glycogen synthetase (UDP glucose-glycogen glucosyltransferase) activity was measured on Tris-EDTA extracts, in the absence and in the presence of 10 mM glucose-6-phosphate, in a reaction mixture having the same composition as the standard medium of Leloir and Goldenberg (1960), except that it was buffered with Tris-acetate, pH 7·5.

Phosphofructokinase, fructosediphosphate, aldolase and lactate dehydrogenase activity determinations were made on K-phosphate extracts, by spectrophotometric procedures. The individual assay procedures were as follows: phosphofructokinase activity was determined by the oxidation of NADH at pH 8·0, in the presence of fructose-6-phosphate, and an excess of crystalline aldolase, triose-phosphate isomerase and α-glycerophosphate dehydrogenase (Ling, Byrne and Lardy, 1955); aldolase by the oxidation of NADH at pH 7·4, in the presence of fructose-1,6-diphosphate, triosephosphate isomerase and α-glycerophosphate dehydrogenase (Wu and Racker, 1959); lactate dehydrogenase by the oxidation of NADH by pyruvate at pH 7·4 (Kornberg, 1955).

Determination of protein.—Total protein nitrogen of the tissue and protein content of the isolated protein fractions were determined by a micro-Kjeldahl method (Markham, 1942). Nitrogen values were converted into mg. of protein by using a factor of 6·25. Protein measurements in whole tissue homogenates and tissue extracts were made by the biuret method or by the Lowry, Rosebrough, Farr and Randall procedure (1951).

Reagents.—Aminoacetonitrile sulphate was obtained from Fluka, Switzerland. Substrates and co-factors for enzymic assays were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. Crystalline enzymes from Boehringer, Germany. Other products were commercial-grade reagents.

RESULTS

Histological Observations

Extensive necrobiotic changes of the muscle fibres cytoplasm, and richness of mononuclear and polymorphonuclear cells, were the main histological features of the grafted tissue, during the first few days after implantation. Soon after the removal of necrotic material by the phagocytic cells had begun, distinguishable myoblasts could be identified throughout the graft. The myoblasts arising in the area of the very small proximal stump of the gastrocnemius and the terminal buds developing at the same site from the excised muscle fibres, contributed little quantitatively, to overall muscle regeneration. The myoblast cells rapidly increased in number from the 3–4th day and, in places, soon developed into myotubes and immature muscle fibres, the latter process becoming more extensive and generalized at about 2–3 weeks. Under conditions of unrestrained growth of fibrillar collagen, the developing muscle cells exhibited a remarkable tendency to become aligned in rows, parallel to the main axis of the limb (Fig. 1). On the other hand, the impaired formation of a guiding collagen framework, following administration of AAN to the rats, appeared to be responsible for the somewhat exaggerated formation of randomly oriented, interweaving bundles of myoblasts, myotubes or muscle fibres (Fig. 2). Though some of the regenerated skeletal muscle fibres showed a continuous gain in girth, still the majority appeared to undergo an atrophic process, during the late periods after implantation, and at 2 months (Fig. 4) they were considerably thinner than at one month (Fig. 3).

These atrophic changes were associated with the production of fibrous connective tissue (Fig. 4). It is of interest that the prolonged administration of AAN at a low dosage to the rats, both inhibited the formation of connective tissue and retarded the onset of atrophy in the regenerated muscle fibres.

Effect of Prolonged Treatment with AAN on the Formation of Insoluble Collagen during Regeneration of Grafted Muscle Tissue

Measurements of insoluble collagen were carried out on both the grafted tissue and on the gastrocnemius from the opposite body side of the same rats. The gastrocnemius, freed of its surrounding connective sheath, had an average content of 3·99 mg./g.f.t.wt. (about 2 per cent of total protein), which was not significantly modified, under the experimental conditions used, following administration of AAN to the rats.

Accompanying the regeneration of skeletal muscle fibres, there was, with the untreated rats, an almost linear increase of collagen proteins in the grafted tissue. In agreement with current views on the mechanism of action of lathyrogens (Mielke, Lalich and Angevine, 1957, Hurley and Ham, 1959); the formation of mature collagen was markedly inhibited, from 30 days after implantation onward, by injecting the rats daily with AAN (Fig. 5).

Yield of Regenerating Tissue

At one week after implantation, the total weight of the grafted tissue was about 25 per cent of the weight of the contralateral gastrocnemius, in both untreated and AAN-treated rats. Thereafter, a different behaviour was observed in the 2 groups of animals. In part due to a decrease in the water content of regenerating tissue, its wet weight fell, in the untreated rats, to an average of 17, 11 and 10 per cent of the weight of the intact contralateral muscle, at 15, 30 and 60 days respectively after implantation. The weight of the regenerating tissue from rats injected with the lathyrogenic nitrile was, when expressed on the same percentage basis, approximately unvaried until the 30th day and dropped to about 15 per cent of the weight of the contralateral gastrocnemius at one month.

Nucleic Acid and Protein Composition of Regenerating Tissue

The results of one experiment in which parallel determinations were carried out, of both total nucleic acid and proteins, are reported in Fig. 6 and Fig. 7, in grafted tissues from untreated and AAN-treated rats.

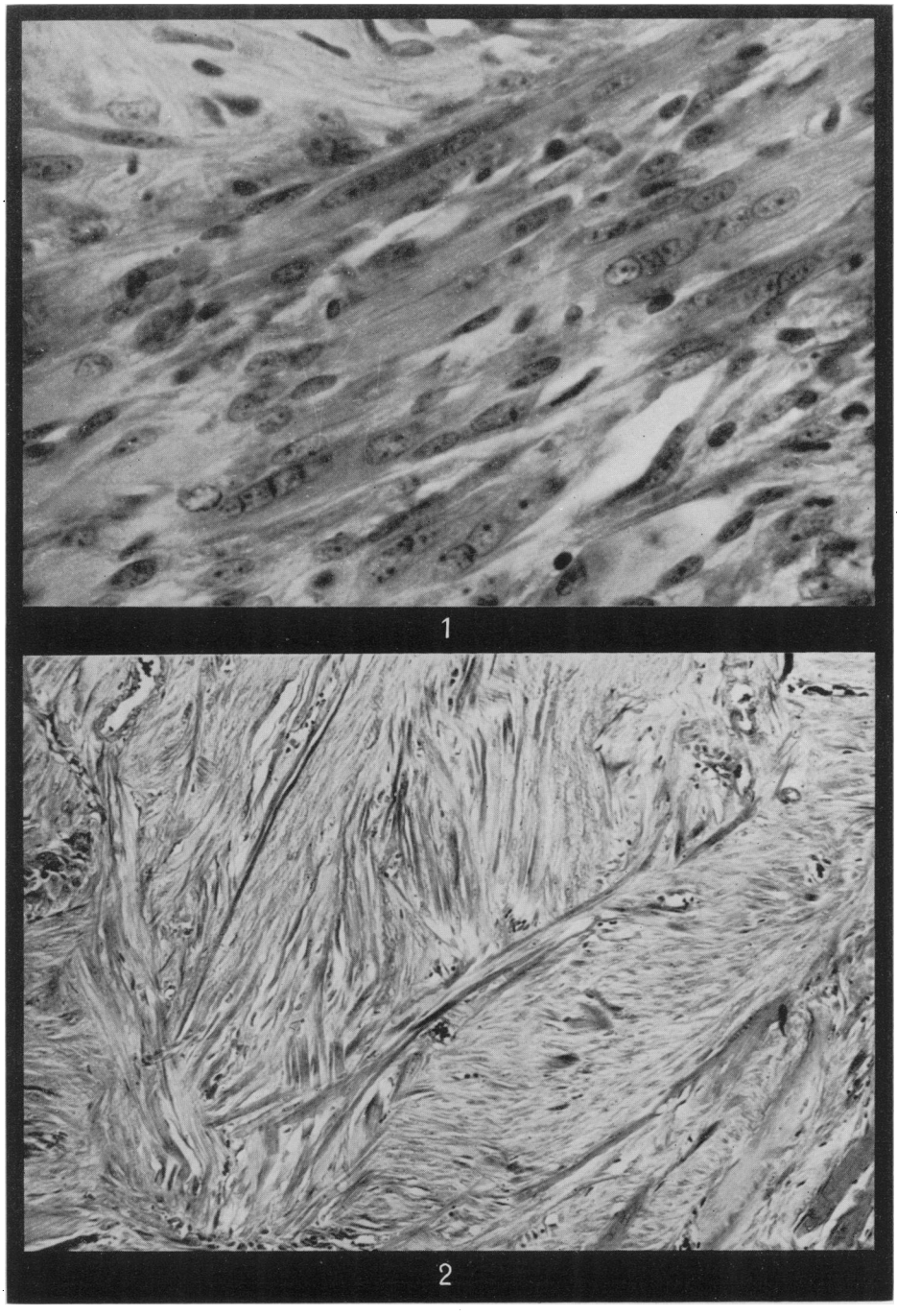
EXPLANATION OF PLATES

FIG. 1.—Area of formation of myotubes and immature muscle fibres in grafted tissue, 7 days after implantation. The host-rat received no treatment. H. and E. ×360.

FIG. 2.—Disorderly arrangement of developing muscle cells at various stages of differentiation. in a rat receiving daily injections of Aminoacetonitrile, at 27 days after implantation. Azan-Mallory. ×90.

FIG. 3.—Trophic appearance of the regenerated skeletal muscle fibres, one month after implantation of the autologous muscle tissue in a rat receiving no treatment. Azan-Mallory. ×225.

FIG. 4.—Atrophic appearance of the regenerated skeletal muscle fibres and excessive growth of connective tissue at 120 days. Other conditions as in Fig. 3. Azan-Mallory. ×225.



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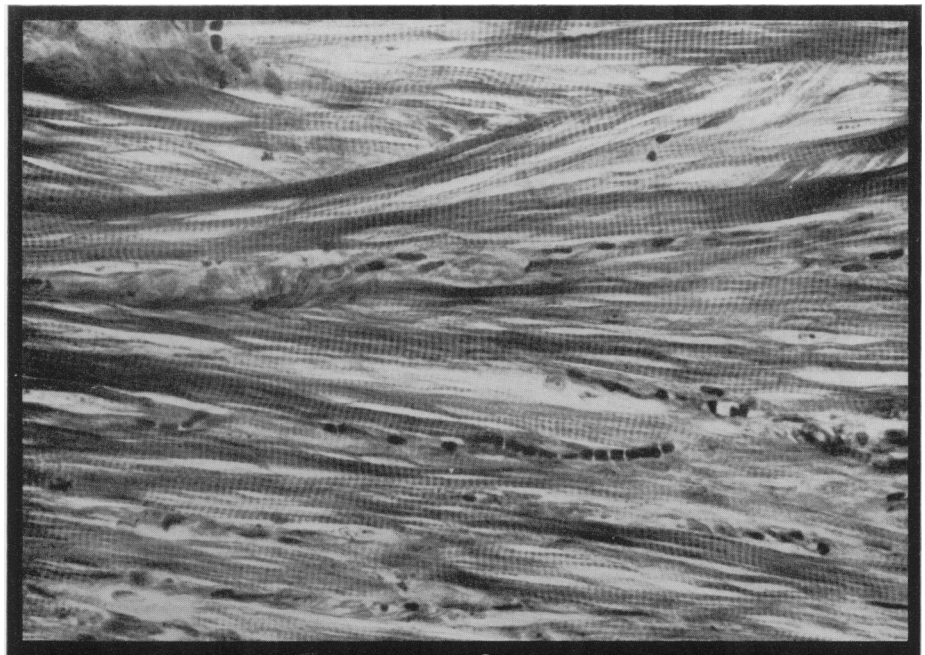

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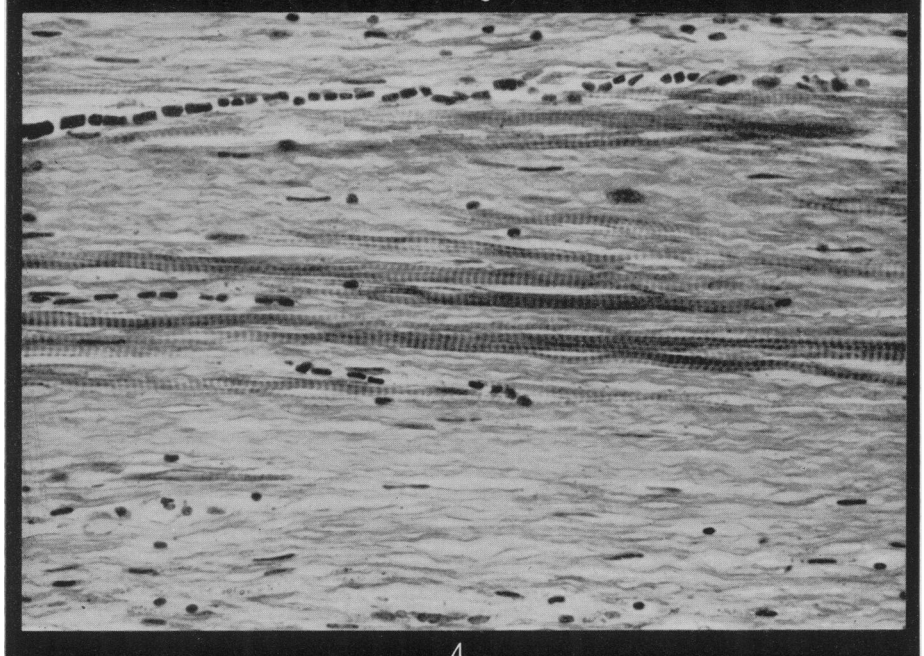

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As the result of the active proliferation of myoblasts, and, to a lesser extent, of connective tissue cells, the concentration of total nucleic acids increased considerably in the early periods after implantation, and reached a maximum at about 15 and 30 days, in the normal and lathyric rats, respectively. The maximal nucleic acid/protein ratio obtained with the grafts from the latter group of animals was also one-third more elevated than in the rats receiving no treatment (Fig. 6).

Due to the degenerative and clearance changes, there was a decrease of contractile proteins in the first week after implantation. At later periods, the relative concentration of contractile proteins in the regenerating tissue varied in a fashion

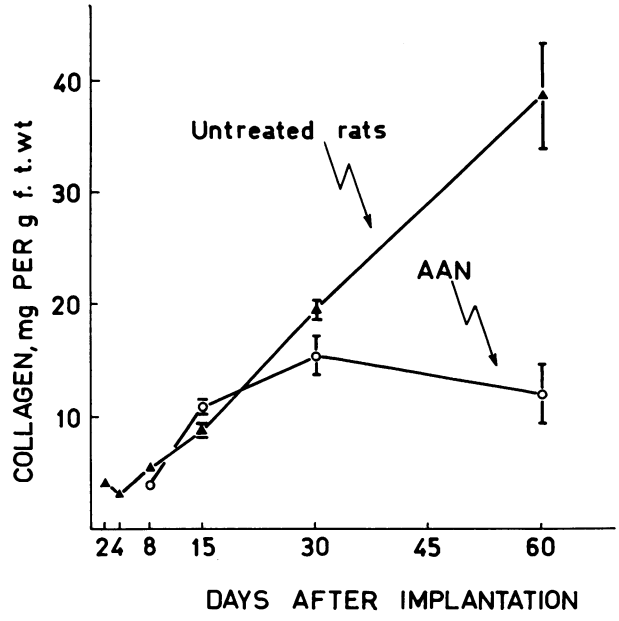

FIG. 5.—Changes of collagen concentration in regenerating tissue from untreated and Aminoacetonitrile-treated rats. Weighed samples of tissue were fractionated by the procedure of Robinson (1952*a*). The collagen content of the residue after alkaline treatment was estimated from hydroxyproline analysis. Each value is the average of 2–7 determinations. Mean values ± S.E.M. are reported when more than 3 determinations were carried out.

which was similar to that observed with total nucleic acids. Only at 1–2 months after implantation, a pronounced difference was noted between the 2 groups of experimental animals. In the untreated rats, the contractile proteins fell rapidly after the initial increase, whereas they decreased more slowly and were still elevated on the 54th day (Fig. 7) in the rats given AAN.

To investigate peculiarities in the behaviour of DNA and RNA during muscle regeneration, these were estimated from ribose and deoxyribose measurements of tissue hot acid extracts from additional groups of experimental animals. The results of these determinations, together with those of total proteins and of contractile proteins are reported in Table I and Table II.

DNA and RNA concentrations increased sharply in the regenerating tissue from both untreated (Table I) and AAN-treated rats (Table II), in the first 2 weeks. Thereafter, a different behaviour was found with DNA and RNA,

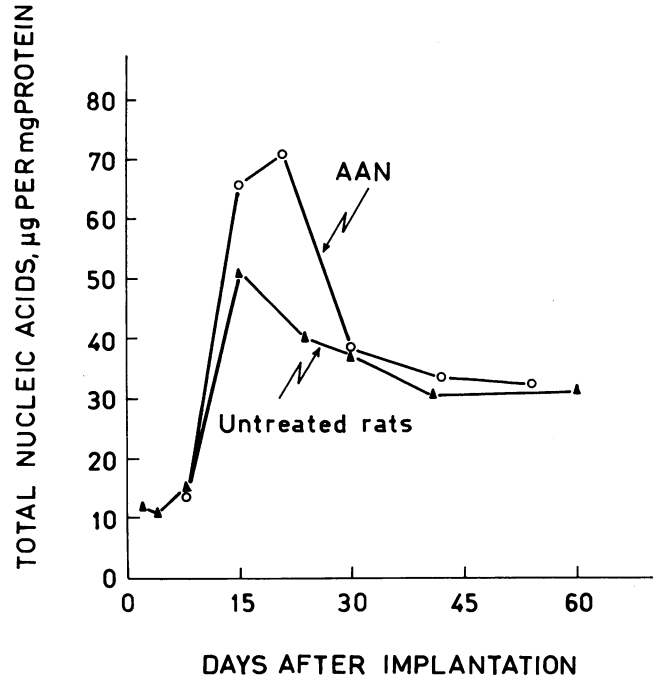

FIG. 6.—Changes of total nucleic acid concentration in regenerating tissue from untreated and Aminoacetonitrile (AAN)-treated rats. Young adult, male Wistar rats from the same stock were the animals used. The animals were divided into 2 groups of 30 animals each. Treatment with AAN was started on one group of rats, 2 weeks prior to the surgical intervention. This was carried out on the same day with all rats. Control rats received no treatment. At any time reported in the Table, 4–6 rats were killed. For chemical analysis, the tissues were removed from 2 or 3 rats and pooled. Total nucleic acid-phosphorus was estimated on hot trichloro-acetic acid extracts. Except for the values at 24 and 41 days for the untreated rats and at 42 days for the rats injected with AAN, all other values reported in the Table are the means of 2 or 3 determinations.

TABLE I.—*Protein and Nucleic Acid Composition of Regenerating Muscle Implants. Untreated Rats*

	Control gastrocnemius m.*	Regenerating muscle		
		15 days†	30 days	60 days
DNA	0·50±0·02 (4)	1·92±0·24 (3)	1·75±0·29 (4)	1·54 (2)
RNA	0·84±0·12 (4)	3·51±0·31 (6)	2·39±0·31 (6)	1·99±0·12 (3)
Total proteins	181·0±10·1 (4)	94·0±6·14 (6)	127·0±10·2 (6)	131·6 (3)
Contractile proteins	102·1±2·75 (4)	54·0±2·9 (6)	39·0±3·3 (6)	43·6 (3)

Experimental conditions : Results are expressed as mg. per g. of fresh tissue ± S.E.M. Number of determinations (each carried out by duplicate analysis on the pooled tissues from several rats) between brackets. Protein nitrogen was determined by a micro-Kjeldahl procedure (Markham, 1942). RNA was estimated from ribose analysis of hot trichloro acetic acid extracts, and DNA from deoxyribose analysis of perchloric extracts (see Methods).

* Contralateral gastrocnemius m.

† Days after implantation.

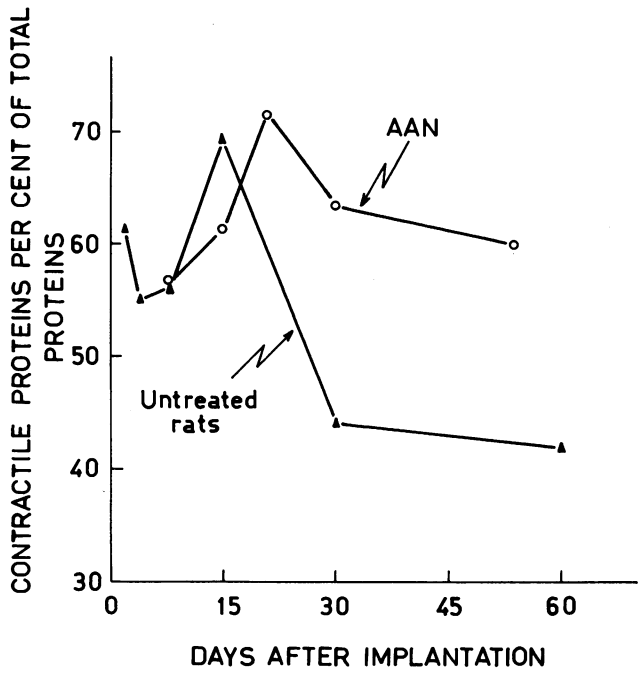

FIG. 7.—Changes of contractile protein concentration in regenerating tissue from untreated and Aminoacetonitrile-treated rats. Weighed portions of the tissues from the same animals of Fig. 6, were fractionated by the procedure of Robinson (1952*a*). Total tissue protein was determined on duplicate amounts of tissue homogenates in distilled water, by the biuret method. Protein nitrogen of the " contractile protein fraction " was determined by a micro-Kjeldahl procedure (Markham, 1942).

according to the treatment of the animals. The levels of DNA, after the initial increases, remained substantially constant during muscle regeneration, and did not appear to be affected, to any large extent, by injecting the lathyrogenic nitrile to the animals.

TABLE II.—*Protein and Nucleic Acid Composition of Regenerating Muscle Implants. Aminoacetonitrile Treated Rats*

	Control gastrocnemius m.*	Regenerating muscle		
		15 days†	30 days	60 days
DNA	0·49 (3)	1·33±0·14 (5)	1·25±0·24 (5)	1·60 (2)
RNA	1·21 (3)	4·05±0·39 (7)	3·64±0·35 (7)	3·50±0·23 (5)
Total proteins	192·0 (3)	120±8·25 (7)	89·5±11·8 (7)	129±14·0 (4)
Contractile proteins	99·9 (3)	49·1±2·9 (7)	56·7±2·2 (5)	62·2±2·6 (4)

Expression of results and experimental conditions were the same as in Table I, except that the rats received daily 50 mg. of AAN/Kg b. wt., by the intraperitoneal route.

* Contralateral gastrocnemius m.

† Days after implantation.

The RNA concentration in the regenerating tissue, on the other hand, dropped rapidly at one to two months in the normal rats (Table I) whereas it declined very slowly in the rats receiving injections of AAN. This is further illustrated in Fig. 8 where the changing RNA/protein ratios are reported, during muscle regeneration. It may also be seen from Fig. 8 that when calculated on a protein basis, the RNA values reached a maximum on about the 15th and 30th day, in the untreated and AAN-treated animals, respectively. The variations of contractile proteins correlated closely with those observed for RNA. From data presented in Tables

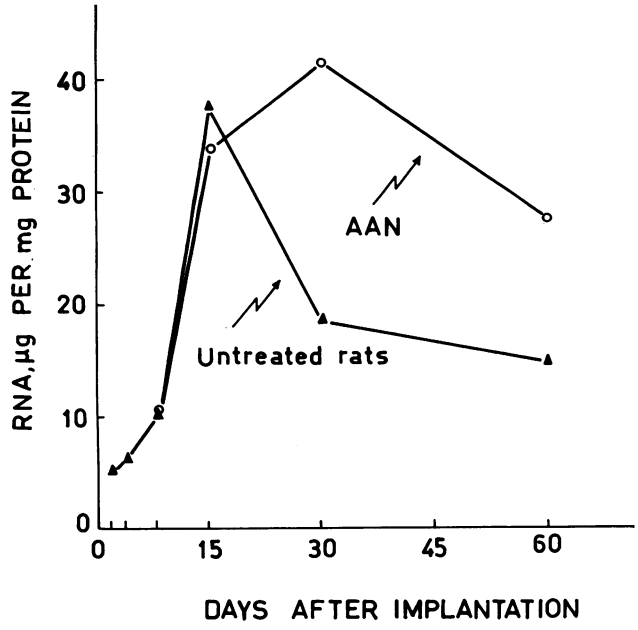

FIG. 8.—RNA : protein ratios in grafts from untreated and Aminoacetonitrile-treated rats. Values at 15, 30 and 60 days were calculated from the data presented in Table I and Table II. Other values are the means of 2–3 determinations on tissues from an additional group of rats.

I and II, it may be noted that the absolute and relative concentrations of contractile proteins were similar at 15 days, but markedly greater at 30 and 60 days, in the regenerating tissue from the lathyric rats as compared with the normal animals. At 30 and 60 days, contractile proteins accounted for 63 and 48 per cent, respectively, of the total tissue proteins in the lathyric rats, whereas they had fallen to only about 30 per cent of total protein in the rats which did not receive treatment with AAN, until the 30th day.

Degree of Maturity of Regenerated Muscle Fibres as Studied from Enzyme Markers

The changing protein composition of developing muscle as been recently re-investigated with the aid of new techniques. It has been shown that, accompanying the pre- and post-natal growth of skeletal muscle fibres, there is a striking

increase of sarcoplasmic proteins, among which the glycolytic enzymes are major constituents (Hartshorne and Perry, 1962 ; Perry and Hartshorne, 1963). Thus certain enzyme activities associated with carbohydrate metabolism may be conveniently used as markers of muscle development (Margreth, unpublished). In order to learn more about the degree of maturity of the regenerated muscle fibres, the activities of glycogen, synthetase, phosphorylase, and of phosphofructokinase, aldolase and lactate dehydrogenase, were measured in extracts of the grafted tissue, at periods corresponding to maximal growth and differentiation. The data in Table III show that regenerating muscle contained about 10 per cent as much total phosphorylase and glycogen synthetase, and 15–20 per cent as much phosphofructokinase, aldolase and lactic dehydrogenase as compared with mature skeletal muscle, when these enzyme activities were expressed per " gramme of fresh tissue ", and even less than that by expressing the results in relation to DNA.

On the other hand, considerable similarities existed, despite peculiarities in the behaviour of individual enzyme activities, between the overall enzymatic composition of regenerating rat muscle, and that of immature muscle from newborn rats, either when fresh tissue or DNA were used as the reference unit (Table III). Regenerating muscle tended to have less of phosphorylase *a* (−AMP)

TABLE III.—*Activity Levels of Enzyme of the Glycogen-Cycle and Embden-Meyerhof Glycolytic Pathway in Regenerating, Neonatal and Adult Skeletal Muscle*

	Activity per g. fresh tissue wt.			Activity per mg. DNA		
	Regenerating* muscle	Neonatal† muscle	Adult‡ muscle	Regenerating muscle	Neonatal muscle	Adult muscle
(1) Glycogen synthetase						
− G–6–P	20·50 (2)	39·37 (2)	176·46 (16)	11·40	16·40	420·00
+ G–6–P	43·00 (2)	72·75 (2)	302·00 (16)	23·95	30·30	719·04
(2) Phosphorylase						
− AMP	65·50 (5)	95·15 (4)	173·05 (16)	36·40	39·80	412·02
+ AMP	95·80 (5)	207·97 (4)	957·50 (16)	53·30	86·65	2279·46
(3) Phosphofructokinase	6·80 (3)	4·10 (4)	33·02 (5)	3·78	1·70	78·61
(4) Aldolase	9·80 (3)	11·31 (4)	68·95 (6)	5·44	4·71	164·16
(5) Lactate dehydrogenase	58·70 (3)	51·00 (4)	368·20 (5)	32·60	21·25	876·66

Enzymes activities are expressed as follows : Glycogen synthetase : μmoles UDP formed/hr. at 37°. Phosphorylase : μmoles P_i formed/10 min. at 30°. Phosphofructokinase : μmoles fructose 1,6-diphosphate formed/min. at room temperature. Aldolase : μmoles triose-P formed/min. at room temperature. Lactate dehydrogenase : μmoles DPNH ox./min. at room temperature. General conditions for enzymic assays were as described in Methods. DNA was estimated by the method of Burton (1956).

* Grafted tissue, 15 to 21 days after implantation. † Muscles of the hind legs and shanks from 5 days old rats. ‡ Gastrocnemius muscle.

Data concerning neonatal and mature skeletal muscle have been obtained in the course of a study on developing muscle (Margreth, unpublished). Number of determinations between brackets.

and of total phosphorylase (+AMP), and less of the 2 forms of glycogen synthetase, acting in the absence and in the presence of glucose-6-phosphate, as compared with muscle from young animals. Aldolase and lactate dehydrogenase activities were in the same range of values in the 2 tissues, whereas phosphofructokinase was 50–100 per cent greater in regenerating muscle than in the neonatal muscle.

It is conceivable that the lower activity ratios, in regenerating muscle, between the 2 enzymes concerned with glycogenolysis and glycogen synthesis, and the enzymes associated with the Embden-Meyerhof route proper, may have resulted from the admixture of a larger proportion of myoblasts, and of fibroblasts to the skeletal muscle fibres in this tissue, as compared with muscle from newborn rats.

DISCUSSION

The histological evidence obtained in the course of this and previous studies (Aloisi, Ferolla and D'Ancona, 1963), supports the " discontinuous " or " embrionic " form (Field, 1960) of muscle regeneration as the main process for the regeneration of skeletal muscle fibres from autologous grafted minces of gastrocnemius in the rat. This mode of regeneration has been recently re-investigated, with the aid of autoradiographic (Bintliff and Walker, 1960 ; Walker, 1963) and electron microscopic techniques (Allbrook, 1962 ; Price, Howes and Blumberg, 1964*a*, *b*) in regenerating muscles following, crush, ischaemic, and cold injury. It appears to involve extensive de-differentiation of the muscle fibres and subsequent formation of myoblasts which derive, exclusively, from the break up of the constituent nuclei of the fibres surrounded by portions of the cytoplasm, which are spared by the degenerative process (Walker, 1963). Still a controversial problem is the way of formation of multi-nucleated myoblasts and myotubes, although several lines of evidence have so far been accumulated, supporting the opinion that these cells arise by fusion of single myoblasts, in both regeneration (Bintliff and Walker, 1960 ; Price, Howes and Blumberg, 1964*b*) and myogenesis (Stockdale and Holtzer, 1961 ; Shafiq, 1963).

By applying quantitative biochemical methods to the study of the development of skeletal muscle fibres in regenerating muscle autografts, we have been able to delineate several stages following the very initial necrobiotic and clearance phenomena.

Active DNA synthesis, RNA synthesis, and synthesis of myofibrillar proteins, were the prominent features of the early phases of regeneration, and appeared to be the biochemical correlates of the extensive proliferation of myoblasts, and of the marked basophila (Pearson, 1962) and richness of ribonucleoprotein particles described in the cytoplasm of these cells, at sites of formation of the early myofilaments (Price, Howes and Blumberg, 1964*b*). Thus, the positive correlations that we could establish in the present study, between changing levels of RNA and of contractile proteins during muscle regeneration, lend further support to the view, purported by the above morphological observations, that these are indeed co-ordinated phenomena, in the early periods of muscle development. The steady increase in concentration of both DNA and RNA, occurring during the first 2 weeks after implantation of muscle tissue in rats, is also in harmony with previous knowledge of the chemical composition of rapidly growing tissues (Davidson and Waymouth, 1944) and bears considerable similarities to the changes observed with embryonic chick muscle during the first week of development (Gluck and Kulowitch, 1964).

At two weeks, on the other hand, DNA had already stopped to increase, and its concentration in the regenerating tissue became established at fairly constant values, throughout the subsequent periods studied. The behaviour observed with

DNA in regenerating muscle autografts, is in conformity with present ideas concerning the mode of multiplication of muscle nuclei in the "discontinuous" form of regeneration. Since it has been shown, by autoradiographic techniques (Bintliff and Walker, 1960; Walker, 1963) that the division of muscle nuclei occurs, mainly at the myoblasts stage of differentiation, it is reasonable that further development of the myoblasts into myotubes and skeletal muscle fibres was accompanied by only small changes in the tissue concentration of DNA.

There is, during embryonic and foetal development of skeletal muscle (Davidson and Waymouth, 1944; Leslie and Davidson, 1951; Gluck and Kulovitch, 1964), and during muscle post-natal growth (Devi, Mukundan, Srivastava and Sarkar, 1963) a marked decrease in concentration of RNA. If the decrease in concentration of RNA, occurring with the regenerating tissue in the late periods after implantation, were to express the further differentiation of the regenerated muscle fibres, this was, however, difficult to reconcile with the behaviour found with contractile proteins which also tended to decrease in their absolute and relative amounts. More pertinent to the interpretation of these findings, may be the observation that the decrease in the concentration of RNA and of contractile proteins was co-incident with the rise of insoluble tissue collagen, occurring between 30–60 days, and was considerably reduced by the administration of AAN to the rats. Thus, it would seem likely that the lower content of RNA and of contractile proteins in the regenerating tissue, in part at least derived from the changing proportions of the muscle phase proper versus intercellular space, in these late periods.

Furthermore, histological evidence showed that there was little cytoplasmic growth with most of the regenerated muscle fibres after one month. The atrophy of the muscle fibres, in the late periods after implantation, could partly be attributed to the competitive growth of fibrous connective tissue (Field, 1960), since it occurred to a lesser extent in the rats given daily injections of AAN. However, the failing formation of myoneural junctions from the growing nerve endings in the grafted area, probably was the main factor responsible for the arrest in development and/or the de-differentiation of the regenerated muscle fibres (Allbrook, 1962).

It seems, then, that the early phase of muscle development is regulated, mainly, by autogenic mechanisms which are insufficient, however, to insure both further progress toward maturity and the maintenance of cell differentiation. In this regard, the pattern of growth and the reversible differentiation generally observed with grafted muscle tissue, bear some similarities to that studied with tissue culture techniques *in vitro* (Konigsberg, McElrain, Tootle and Hermann, 1960; Konigsberg, 1963). Comparative studies of the morphology and biochemistry of muscle development in these different systems, and of muscle pre- and post-natal growth, may provide, therefore, better clues to the understanding of the factors which regulate muscle differentiation at various phases of development. Congruous with the postulated neural influence affecting only the late stages of muscle differentiation (Eccles, 1963), and with the notion that the rate of growth of the soluble, glycolytically active sarcoplasm is slow during muscle development (Dickerson and Widdowson, 1960) and becomes more active only after birth (Hartshorne and Perry, 1962; Perry and Hartshorne, 1963), is the finding reported in this paper that there are comparable levels of enzyme activities concerned with carbohydrate metabolism, in regenerating rat muscle and in muscle from newborn rats.

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SUMMARY

The whole gastrocnemius muscle was removed from one leg of rats. By grafting a mince prepared from the same muscle, in its original anatomical area, it was possible to obtain extensive regeneration of muscle tissue, in amounts amenable to biochemical analysis.

The proliferation of myoblasts, and the formation of myotubes and skeletal muscle fibres could be traced by the changes in the tissue concentration of DNA, RNA, and contractile proteins. In conjunction with these quantitative determinations, measurements were also carried out of insoluble collagen. The effect exerted on its rate of formation by the lathyrogen, aminoacetonitrile, was studied, by administering this compound daily to the rats. The general features of muscle regeneration under these and under the basic conditions were compared.

Definite relationship could be established, both in untreated and AAN-treated rats, between concentrations of total RNA and contractile proteins, during development of the regenerating muscle cells. On account of its low content of enzymes related to glycogen synthesis and glycogenolysis, the average regenerated muscle fibre appeared unable, under the prevailing conditions, to grow to complete maturity and resembled closely the immature muscle from newborn rats.

Atrophic (*i.e.* de-differentiation) changes occurred in the regenerated muscle fibres, in the late stages after implantation. These modifications were probably the result of the altogether insufficient formation of myoneural junctions, and of the excessive growth of fibrous connective tissue. The daily administration of aminoacetonitrile to the host-rats, inhibited considerably both the production of insoluble collagen, and the onset of atrophy in the regenerated muscle fibres.

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